5.0 DATA ON *IN VITRO* ER TA ASSAYS

5.1 Introduction

Data and methodology information were collected from 86 publications and two submitted unpublished reports on substances that had been evaluated for their ability to act as an ER agonist and/or antagonist *in vitro* in a reporter gene or cell proliferation assay. Where provided, the specific information extracted for each tested substance included its name, source, purity, methodological details, and relevant data. If available, a CASRN was identified for each substance. This identifier was obtained from various sources, including the source publication, the National Library of Medicine's ChemID database, and *The Merck Index*. Chemical name synonyms were collected for substances that were identified in the literature by more than one name, and for substances where the name used in the publication may have been different from the generic name. All substances with the same CASRN are listed under the same name, usually the common name, regardless of the name that was used in the original publication. No attempt was made to identify the source and purity of a substance if the investigators did not provide such information. **Appendix C** provides information on the names, synonyms, CASRN, and chemical/product class, if identified, for each substance. **Appendix D** contains the *in vitro* ER TA data, which is sorted by chemical name and then type of assay, both in alphabetical order.

5.2 Availability of Detailed *In Vitro* ER TA Protocols

The Methods sections in the *in vitro* ER TA publications and the two unpublished reports provided various levels of detail. Relevant method parameters were extracted from each source and summarized in **Appendix A**. Details about the following method parameters are included in the Appendix to the extent this information was available:

- *Characteristics of cell line or yeast strain* (e.g., name of cell line/yeast strain and the source of the cell line);
- *Plasmids used to transfect cells* (e.g., ER source, ER expression vector, reporter vector, endpoint measured, plasmid transfected for cell toxicity measurements, and transfection method [i.e., whether stable or transient] and procedures);
- *Preparation of cells for assay* (e.g., growth of cells before transfection, plating time prior to treatment of cells with a test substance); and

- *Assay type* (e.g., reporter gene or cell proliferation assay, agonism and/or antagonism, solvent(s) used, test substance concentration, test substance exposure duration, reference estrogen and its concentration, number of replicates per experiment, number of times assay was repeated).

5.3 Availability of *In Vitro* ER TA Data

In vitro ER TA data for a total of 698 unique substances are included in **Appendix D**. In this Appendix, italicized values for quantitative measures of agonist or antagonist activity indicate values estimated from graphically presented data. The following *in vitro* ER TA reporter gene and cell proliferation assays were used to generate the data included in this BRD.

1. *Yeast ER TA reporter gene assays:* Data generated using 22 different *in vitro* yeast ER TA reporter gene assays involving at least 13 different strains of yeast (most likely *S. cerevisiae*) are included in this BRD. **Table 2-1** provides information, when specified in the publication or the report, on the yeast strain used, the ER source, the ER subtype, the plasmids transfected, and the corresponding designation used in this BRD. Strains stably transfected with ER (predominantly hERα) and a β-galactosidase expression vector were used in these studies.

2. *Mammalian cell ER TA reporter gene assays:* As provided in **Appendix D**, 12 different mammalian cell lines and several variants were used in *in vitro* ER TA reporter gene assays to assess the ability of a test substance to express ER agonist or antagonist activity (see **Table 2-2**). The MCF-7 cell line, derived from a human breast cancer, has been used most frequently; other human cell lines used include BG-1 (human ovarian carcinoma), HEC-1 (human endometrial tumor), HEK293 (human embryonal kidney), HeLa (human cervical tumor), HepG2 (human liver tumor), Ishikawa (human endometrial tumor), MDA-MB-231 (human breast tumor), T47D (human breast tumor), and ZR-75-1 (human breast tumor). In addition, CHO-K1 (Chinese hamster ovary), COS-1 (monkey kidney cells), and ELT-3 (rat uterine leiomyoma cells) have been used. The majority of these assays used a transiently transfected *Luc* reporter gene; however, stably transfected *Luc* or transiently/stably

transfected *CAT* genes were also used. Some laboratories transfected the cells with the β-*gal* gene as an internal control to assess transfection efficiency and cell toxicity.

3. *Mammalian cell ER TA proliferation assays:* Three cell lines and several variants that exhibit increased levels of cell proliferation in response to estrogen have been used to measure the potential estrogenicity of substances. The cell proliferation assays included in this BRD, organized by cell line, are provided in **Table 2-3**. The majority of these assays used the MCF-7 cell line.

In studies that evaluated the potential agonism of a substance in an *in vitro* ER reporter gene assay, enzyme (i.e., luciferase; CAT; β-galactosidase) activity was used as a measure of ER-induced TA. To assess agonism potency, reporter gene enzyme levels induced by the test substance were typically compared to those produced by the reference estrogen, predominantly 17β-estradiol. The quantitative results from these *in vitro* ER TA studies were most commonly presented in terms of relative activity. However, the definition of relative activity varied greatly among the reports. Relative activity was expressed as:

- Miller Units in some yeast reporter gene assay studies (see **Section 2.3.2**);
- The ratio of the response of the reference estrogen to that of the test substance (sometimes termed relative potency and calculated as [EC_{50} 17β-estradiol/EC_{50} test substance] x 100);
- Percent maximal response;
- The concentration of the test substance that produced a certain percent response relative to the reference estrogen;
- The concentration of test substance that produced a specified fold-induction (e.g., 10-fold induction of enzyme activity) over background; and
- Fold induction of enzyme activity produced by the test substance relative to the activity in the untreated controls.

These quantitative measures of agonism, if available, were extracted from the publications and reports. In reports where an EC_{50} value was not provided but dose response data were presented, the EC_{50} values of the test substance and the reference estrogen were estimated. Such data are provided in **Appendix D** in the "Agonism (Relative Activity)" and the "Agonism (EC_{50} μM)"

columns. Normalizing these values for comparison across assays was not attempted. Instead, data from each study was assigned a qualitative response of positive or negative for the particular assay system and is provided in **Appendix D** in the "Agonism (Qualitative)" column.

ER cell proliferation studies reported results as cell number, foci/cm^2, EC_{50} values, cell growth relative to hormone free control, increase in protein or DNA content, and fold increase in cell number relative to vehicle control. Data from each study was assigned a qualitative response of positive or negative for the particular assay system, as shown in **Appendix D** in the "Cell Growth" column.

The antagonism studies that used reporter gene expression or cell proliferation measured the inhibition of reference estrogen-induced enzyme activity or cell growth, respectively, by the test substance. The IC_{50} value was often presented as a measure of response. These values are summarized in **Appendix D** in the "Antagonism (Relative Activity)" column. In reports where an IC_{50} value was not provided but dose response data were presented, the IC_{50} values of the test substance and the reference estrogen were estimated. These estimated IC_{50} values are italicized in **Appendix D**. Where an IC_{50} value was not reported or a dose-response curve was not presented, test substances were assigned a qualitative response of "positive" or "negative" in the assay system used, as shown in **Appendix D** in the "Antagonism (Qualitative)" column.

5.4 *In Vitro* ER TA Assay Results for Individual Substances

Of the 698 substances tested in the *in vitro* ER TA assays considered in this BRD, 534 were tested in mammalian cell/yeast reporter gene agonism assays and 174 were tested in mammalian cell/yeast reporter gene antagonism assays. The substances tested in five or more mammalian cell/yeast reporter gene assays are provided in **Table 5-1**. Only 42 substances were tested for agonism in five or more mammalian cell/yeast reporter gene assays, while only eight substances were tested in five or more mammalian cell/yeast reporter gene antagonism assays. The greatest number of different mammalian cell/yeast reporter gene agonism assays used to test the same substance was eighteen, for Bisphenol A. The greatest number of different mammalian cell/yeast reporter gene antagonism assays used to test the same substance was thirteen, for ICI 182,780. More than 50% of substances tested in mammalian cell/yeast reporter gene agonism

assays (317 of 534 substances; 59.4%) were tested in one assay only. Similarly, about half (76 of 174; 43.7%) of the substances tested in the reporter gene antagonism assays were tested in one assay only.

A total of 312 substances were tested in ER cell proliferation agonism assays; 67 were tested in ER cell proliferation antagonism assays. Only 47 substances were tested for ER agonism in two or more cell proliferation assays, while only eight were tested in two or more ER cell proliferation antagonism assays (**Table 5-2**). The greatest number of different cell proliferation agonism assays used to test the same substance was four, for bisphenol A, bisphenol A dimethacrylate, and estrone, while the greatest number of different cell proliferation antagonism assays used to test the same substance was three, for ICI 182,780.

A majority of the substances tested in ER cell proliferation agonism assays (265 of 312 substances; 85%) were tested in one assay only. Fifty-nine of the 67 (88%) substances tested in ER cell proliferation antagonism assays were tested in one assay only.

5.5 Use of Coded Chemicals and Compliance with Good Laboratory Practice (GLP) Guidelines

Based on the available information, it appears that none of the *in vitro* ER TA studies included in this BRD used coded chemicals. Only Xenobiotic Detection Systems, Inc. stated that its studies were conducted in compliance with GLP guidelines.

Table 5-1 Substances Tested in Five or More *In Vitro* Mammalian Cell or Yeast ER TA Reporter Gene Assays

Substance	Number of Agonism Assays	Number of Antagonism Assays
Bisphenol A	18	
Diethylstilbestrol	17	
Methoxychlor	17	
Genistein	16	5
o,p'-DDT	14	
ICI 182,780	9	13
HPTE	12	5
4-Hydroxytamoxifen	10	12
p-Nonylphenol	11	
Zearalenone	11	
Coumestrol	10	5
2',3',4',5'-Tetrachloro-4-biphenylol	9	
2',4',6'-Trichloro-4-biphenylol	9	
Biochanin A	9	5
Daidzein	9	
Tamoxifen	9	8
Dibutyl phthalate	8	
Testosterone	8	
4-*t*-Octylphenol	7	
Bis(2-ethylhexyl)phthalate	7	
Butyl benzyl phthalate	7	
Dieldrin	7	
Estrone	7	
Kepone	7	
p,p'-DDT	7	
Equol	6	
Formononetin	6	
o,p'-DDD	6	
p,p'-DDE	6	
α,β-Endosulfan	6	
ICI 164,384	5	6
17α-Estradiol	5	
17α-Ethinyl estradiol	5	
5α-Dihydrotestosterone	5	
Atrazine	5	
Dexamethasone	5	
Ethanol	5	
Ethinyl estradiol	5	
Naringenin	5	
Nonylphenol	5	
α-Zearalanol	5	
β-Zearalenol	5	

Abbreviations: DDT = Dichlorodiphenyltrichloroethane; *o,p'*-DDD = 1,1-Dichloro-2-(*o*-chlorophenyl)ethane; *p,p'*-DDE = 1,1-Dichloro-2,2-bis[*p*-chlorophenyl]ethylene; HPTE = 2,2-Bis-(*p*-chlorophenyl)-1,1,1-trichloroethane

Table 5-2 Substances Tested in Two or More *In Vitro* ER TA Cell Proliferation Assays

Substance	Number of Agonism Assays	Number of Antagonism Assays
Bisphenol A	4	
Bisphenol A dimethacrylate	4	
Estrone	4	
Butyl benzyl phthalate	3	
ICI 182,780		3
17α-Estradiol	2	
17α-Ethinyl estradiol	2	
2-Chlorobiphenyl	2	
2,2',5,5'-Tetrachlorobiphenyl	2	2
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	2	
2,4-Dichlorophenol	2	
2,5-Dichloro-4'-biphenylol	2	
2,5-Dichlorobiphenyl	2	
2',4',6'-Trichloro-4-biphenylol	2	
2-Chlorobiphenyl	2	
3,3',5,5'-Tetrachlorobiphenyl	2	2
3,5-Dichloro-4'-biphenylol	2	
3,5-Dichlorobiphenyl	2	
4-Chlorobiphenyl	2	
Apigenin	2	
Bis(2-ethylhexyl)phthalate	2	
Butylated hydroxyanisole	2	
Butylated hydroxytoluene	2	
Chlorpyrifos	2	
Coumestrol	2	2
Dibutyl phthalate	2	
Dicofol	2	
Dieldrin	2	
Diethyl phthalate	2	
Diethylstilbestrol	2	
Dihexyl phthalate	2	
Diisobutyl phthalate	2	
Diisodecyl phthalate	2	
Diisononyl phthalate	2	
Dimethyl sulfoxide	2	
Di-*n*-butyl phthalate	2	
Ditridecyl phthalate	2	
Estriol	2	
Ethanol	2	
Flavone	2	
Genistein	2	2
Glabridin	2	2
Heptachlor	2	

Table 5-2 Substances Tested in Two or More *In Vitro* ER TA Cell Proliferation Assays (continued)

Substance	Number of Agonism Assays	Number of Antagonism Assays
Octylphenol	2	
Raloxifene	2	2
Tamoxifen	2	2
Zearalenone	2	
α-Endosulfan	2	
α-Zearalanol	2	
β-Endosulfan	2	